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The chymase-angiotensin system in humans: Biochemistry, molecular biology and potential role in cardiovascular diseases

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Y LIAO, A HUSAIN. The chymase-angiotensin system in humans: Biochemistry, molecular biology and potential role in cardiovascular diseases. Can J Cardiol 1995;11(Suppl F):13F-19F. Angiotensin I-converting enzyme inhibitors are highly effective in the treatment of cardiovascular diseases; however, the relationship among the antihypertensive effects of ACE inhibition, inhibition and plasma angiotensin II levels is complex. During chronic therapy with ACE inhibition, plasma angiotensin II levels return to normal despite continued antihypertensive effect. Recent studies show that angiotensin I to angiotensin II in tissues can proceed despite complete inhibition of ACE. In the search for a potential ACE inhibitor resistant angiotensin II-forming enzyme activity in human heart tissue, chymase was identified as a major angiotensin II-forming enzyme. In primates, chymase-like angiotensin II-forming activity is localized in a number of tissues including the heart, blood vessels and lungs. Within the human heart, mast cells and endothelial cells are the sites of synthesis and storage of chymase, but a high level of chymase activity is found in the cardiac interstitium, associated with the extracellular matrix. Mammalian chymases may be divided into two distinct structural groups. α chymases, such as human chymase, are highly specific and efficient angiotensin II forming enzymes. β-chymases, including rat mast cell chymases, have a broad substrate specificity like chymotrypsin and are poor angiotensin II forming enzymes. In humans and baboons only a single chymase gene can be identified. By using an angiotensin I analogue that is selectively converted to angiotensin II by chymase and not ACE, a function for chymase in angiotensin II formation has recently been demonstrated in conscious baboons. Because of the deleterious growth effects of angiotensin II on the heart and blood vessels, the potential for incomplete suppression of tissue angiotensin II

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FIVE DECADES OF RESEARCH ON THE renin-angiotensin system has led to the concept that the enzymes renin and angiotensin I-converting enzyme (ACE) play pivotal roles in the sequential conversion of angiotensinogen to angiotensin I and of angiotensin I to angiotensin II (1). Numerous pharmacological studies have revealed that the octapeptide hormone angiotensin II greatly influences blood pressure homeostasis, hydromineral balance and tissue remodelling (1,2). That these effects of angiotensin II are important in the progression of cardiovascular diseases has been reiterated by the recent success of ACE inhibitor therapy in the treatment of patients with hypertension, left ventricular dysfunction after myocardial infarction (3,4), asymptomatic left ventricular dysfunction (5) and chronic, symptomatic heart failure (6-8).

In 1982, Biollaz et al (9) reported that during chronic antihypertensive therapy with the ACE inhibitor enalapril, plasma angiotensin II levels were not suppressed despite effective normalization of blood pressure and a greater than 90% inhibition of plasma ACE activity. Because of these appar-

formation [illegible] inhibitor therapy is hypothesized that the inhibition of chymase and ACE may be more efficacious in the treatment of cardiovascular diseases than ACE inhibitor therapy alone.

Key Words: [illegible] inhibitors, chymase, angiotensin system, [illegible]

Système [illegible] chez l'humain : biochimie, biologie moléculaire et rôle [illegible] dans les maladies cardiovasculaires

RÉSUMÉ : [illegible] de l'enzyme de conversion de l'angiotensine (ECA) sont très efficaces dans le traitement des maladies cardiaques. Toutefois, le rapport entre les effets antihypertensifs des inhibiteurs de l'ECA, l'inhibition de l'ECA et les taux plasmatiques d'angiotensine est complexe. Durant le traitement prolongé par inhibiteurs de l'ECA, les taux plasmatiques d'angiotensine II redeviennent normaux malgré l'effet antihypertensif persistant. De récentes études révèlent qu'une conversion de l'angiotensine I en angiotensine II au niveau tissulaire peut se produire malgré l'inhibition complète de l'ECA. Dans le cadre de recherches sur l'existence potentielle d'une activité enzymatique responsable de la formation de l'angiotensine II résistante aux inhibiteurs de l'ECA dans le tissu cardiaque humain, la chymase a été identifiée comme enzyme importante. Chez les primates, une substance responsable de la formation de l'angiotensine semblable à la chymase est localisée dans un certain nombre de tissus, y compris le coeur, les vaisseaux sanguins et les poumons. Dans le coeur humain, les mastocytes et les cellules endothéliales sont le lieu de synthèse et de stockage de la chymase, mais une forte proportion de la chymase sécrétée se trouve également dans l'interstice cardiaque, associée à la matrice extracellulaire. Les chymases chez les mammifères se divisent en deux groupes structurels distincts, les chymases α et les chymases β. Les α-chymase, comme la chymase chez l'humain, sont des enzymes très spécifiques et très efficaces pour la formation de l'angiotensine II. Les β-chymases, y compris les chymases de plusieurs espèces de rats et de souris, ont une vaste spécificité quant à leur substrat, comme la chymotrypsine, et elles ne forment pas d'angiotensine II. Chez les humains et les babouins, seul le gène unique [illegible] alpha peut être identifié. À l'aide d'un analogue [illegible] angiotensine I qui est sélectivement converti en angiotensine II par la chymase et non par l'ECA, la formation fonctionnelle d'angiotensine II dépendante de la chymase a récemment été démontrée chez des babouins conscients. À cause de l'effet délétère de la croissance de l'angiotensine II au niveau du coeur et des vaisseaux sanguins et du risque de suppression incomplète de la formation tissulaire de l'angiotensine II durant le traitement par inhibiteurs de l'ECA, il est supposé que l'inhibition de la chymase et de l'ECA puisse être plus efficace dans le traitement des maladies cardiovasculaires que le traitement par inhibiteurs de l'ECA seul.

ently dichotomous results, these investigators concluded that this degree of ACE inhibition was not sufficient to block effectively the renin-angiotensin system. The concept of incomplete ACE inhibition during chronic ACE inhibitor therapy was strengthened as other investigators, using more sophisticated high performance liquid chromatography methodology for the measurement of angiotensin II (10), confirmed the observations of Biollaz et al (9). In 1979, Cornish et al (11) observed that angiotensin I-dependent vasoconstriction in hamster cheek pouch blood vessels was partially inhibited by ACE inhibition but completely inhibited by pretreatment of the vessel preparation by angiotensin II antiserum or by angiotensin II receptor antagonist; they suggested that a functional conversion of angiotensin I to angiotensin II in blood vessels may be effected by a novel ACE activity. Similar findings were also observed in the cheek pouch microcirculation of the adult monkey (*Macaca fascicularis*) (12). To some investigators, this conclusion allowed an alternative explanation of the findings presented by Biollaz et al (9), ie, that incomplete suppression of plasma angiotensin II levels during chronic ACE inhibitor therapy may be due to the presence of a pathway for the conversion of angiotensin I to angiotensin II that is distinct from ACE.

In 1984, using an isolated organ bath, Okunishi et al (13) convincingly demonstrated that complete suppression of an angiotensin I-mediated vasoconstriction of the dog coronary vessel needed inhibition of ACE and chymotrypsin-like activities. In 1989, our studies with Langendorff preparations of hamster heart demonstrated a substantial angiotensin I-mediated positive inotropic effect in the presence of ACE inhibition (14). In the search for a potential ACE inhibitor-resistant, angiotensin II-forming enzyme activity in human heart tissue, we identified chymase as a major angiotensin II-forming enzyme in the heart (15,16). Subsequent studies by our group and by others led to the concept of the chymase-angiotensin system, which may be important in regulating tissue angiotensin II levels (17), and more recent studies implicate a role for chymase-dependent angiotensin II formation in vessel disease.

This review examines recent advances in our understanding of the biochemistry and molecular biology of human chymase, evaluates the evidence for the existence of a functional chymase-dependent angiotensin II-forming pathway in primates, and outlines potential therapeutic implications of the chymase-angiotensin system.

LOCALIZATION OF CHYMASE IN THE HEART AND VESSELS

Chymase-like immunoreactivity is localized in the cardiac interstitium and is probably associated with the interstitial extracellular matrix (18). Electron microscopic (EM)-immunocytochemical and in situ hybridization studies indicate that the mast cells and endothelial cells within the human heart are the sites of elaboration of chymase (18). Other mammalian chymases studied have also been shown to be stored in an active form in secretory

granules of the mast cell (19,20), but so far only endothelial cells from human heart have been shown to contain chymase mRNA and chymase-like immunoreactivity (18). In these cardiac endothelial cells, chymase-like immunoreactivity is present in Weibel-Palade bodies. Recent studies indicate that secretion of the von Willebrand factor stored in Weibel-Palade bodies is polarized, with the bulk of the secretion occurring in the basolateral direction (21), suggesting that chymase will also be released basolaterally. Indeed, EM-immunocytochemical studies indicate the presence of chymase-like immunoreactivity in the basolateral region of endothelial cells (18).

Human chymase is a highly basic enzyme. Molecular modelling studies of human chymase indicate the presence of several positively charged residues on the surface of this enzyme (22). It has been suggested that in the case of rat chymase 1 such positively charged residues play a role in binding the enzyme to heparin or to other sulphated proteoglycans and glycosaminoglycans found in secretory granules, and to the extracellular matrix (23). The highly basic nature of human chymase is common to several proteinases found in mast cell granules, including cathepsin G (24). It is also known that when these mast cell proteinases are bound to heparin, they are relatively resistant to proteolytic degradation and to inactivation by the plasma serine proteinase inhibitor (18,25). Thus, chymase in the heart may be relatively stable and probably remains active after binding to the extracellular matrix.

In contrast, in normal human cardiac ventricles, ACE is chiefly localized on the luminal surface of endothelial cells (26). The difference in the localization of ACE and chymase in the human heart and vessels may indicate that chymase-dependent angiotensin II formation is more prevalent in the myocardial interstitium and the adventitial and medial regions of vessels, whereas ACE-dependent angiotensin II formation is more significant in the lumen of the vessel. Consistent with these observations, Okunishi et al (27) observed that in the dog aorta the major angiotensin II-forming activity is chymase-like in adventitial homogenates and is ACE-like in endothelial homogenates.

Recently, in humans (18) and baboons (28), chymase-like angiotensin II-forming enzyme activity has been identified in a number of tissues in addition to the heart and blood vessels, namely the stomach, colon, uterus and lungs.

BIOCHEMISTRY, ENZYMOLOGY AND REGULATION OF HUMAN CHYMASE

Biochemistry of human chymase: Human chymase (*chymotrypsin-like proteinase*), isolated from the heart (16) and skin (29), is a glycoprotein with an apparent molecular weight of about 30 kDa. The human chymase (*chm*) gene and cDNA have been cloned (30). Based on the difference in primary structure of human chymase deduced from its cDNA structure and the structure of the mature enzyme determined after N-terminal sequencing, human chymase appears to be synthesized as a preproenzyme and post-translationally modified (30). cDNA expression studies in COS-1 cells indicate that the 19 residue signal peptide of human chymase is excised cotranslationally (31). The resulting proenzyme, which contains a two-residue activation peptide, is inactive. It is known that the active enzyme is stored in the mast cell granule complexed with heparin. In the mast cell granule, the two-residue activation peptide is excised by dipeptidylpeptidase-I to activate the zymogen (32). Recently, the mechanism of preprochymase activation was studied by using recombinant prochymase in a model system (33). It appears that a high affinity interaction between heparin and prochymase allows the two-residue activation to be cleaved by dipeptidylpeptidase-I and that a conserved glutamic acid in the activation peptide is necessary for this heparin effect. Following propeptide cleavage, capture of the newly generated N-terminus by an 'activation groove' on the enzyme activates the enzyme.

Chymase isoenzymes: Human chymase has several structural similarities, as well as important dissimilarities, with other mammalian chymases. Similarities include a conserved acidic two-residue activation peptide, and the presence of a catalytic triad (serine-184, histidine-47 and aspartic acid-91) (30). Mammalian chymases may be divided into two distinct groups (22), α and β, based on multiple sequence alignment of their polypeptide sequences. α-chymases include human chymase, dog chymase and mouse chymase-5. β-chymases include mouse chymase-1, -2, -4 and -L, and rat chymase-1 and -2. Kinetic studies suggest that α- and β-chymases may also differ in their substrate specificity. Studies with peptide substrates have indicated that human chymase has a restricted substrate specificity and is a highly efficient and specific angiotensin II-forming enzyme (16,34). Dog chymase appears to have a substrate specificity similar to that of human chymase. Rat chymase-1, on the other hand, has a wider substrate specificity, similar to that of chymotrypsin, and has been postulated to play a role in parasite expulsion (35) and angiotensin II degradation (36).

Nature of the high substrate specificity of human chymase: Human chymase, isolated from the heart, rapidly converts angiotensin I to angiotensin II and the angiotensin I C-terminal dipeptide His-Leu (K_m= 60 μM; k_{cat}=160/s); angiotensin II, however, is not degraded, even after prolonged incubations with human chymase (16). Human chymase is an endopeptidase since it converts angiotensin I and human tetradecapeptide renin substrate to angiotensin II by hydrolyzing the Phe^8-His^9 bond in both these peptides (16). However, unlike tonin (37), cathepsin G (38) and kallikrein (39), human chymase (16) does not form angiotensin II from angiotensinogen. When angiotensin I is the substrate, the specificity constant (k_{cat}/K_m) for human heart chymase is higher than that for human ACE or rat tonin, and much higher than that for human cathepsin G (16).

The process of polypeptide catalysis by chymases, as well as other serine proteinases, may be divided into two steps: selection of the scissile bond and

catalysis. The mechanism of catalysis per se is common to all chymases and involves the formation and hydrolysis of an acyl enzyme intermediate in which the active site serine, histidine and aspartic acid all play critical roles. However, there are many differences, as well as similarities, in the way the scissile bond is selected by chymotrypsin and chymotrypsin-like proteinases. Chymotrypsin places a very high emphasis on the P_1 positioned amino acid (either tyrosine, tryptophan or phenylalanine) of peptide substrates but considerably less emphasis on the amino acids surrounding the P_1 residue. However, the major determinant of specificity in chymotrypsin, as well as in β-chymase, is the primary specificity pocket (S_1). Rat chymase 1 shows a high preference for phenylalanine, tyrosine and tryptophan in the P_1 position, but much less emphasis is placed on the extended substrate-binding site (ie, interactions other than the P_1/S_1 interaction) (36,40). For the selection of the scissile bond, human chymase also shows a high preference for a hydrophobic aromatic amino acid in the P_1 position, but needs additional determinants (34). At the S_2 subsite there is a significant preference for Pro over hydrophobic or hydrophillic amino acids. There is no clear preference for hydrophobic or hydrophillic amino acids at the S_1' and S_2' subsites, but substrates containing P_1' Pro are not hydrolyzed and substrates containing P_2' Pro are poorly hydrolyzed. An increasing reduction in reactivity occurs when the P position amino acids in angiotensin I are deleted sequentially from the N-terminus. An increase or decrease in the length of the His-Leu leaving group also produces a marked decrease in reactivity. No single determinant in angiotensin I is preeminently required for efficient catalysis, but several factors acting synergistically appear to be important. Thus, it has been proposed that ideal substrates for human heart chymase should contain the structure nXaa-Pro-[Phe, Tyr, or Trp]-Yaa-Yaa, where $n \leq 6$; Xaa = any amino acid; and Yaa = any amino acid except proline (34). This structure exists in angiotensin I and neurotensin, both of which are good substrates for human chymase (34).

Transcriptional regulation of chymases: Based on patterns of proteinase expression there is now strong evidence for the presence of two types of mast cells in humans, MC_{TC} and MC_T (41). The MC_{TC} type of human mast cells store and secrete chymase and the trypsin-like proteinase tryptase, whereas the MC_T type of human mast cells store and secrete tryptase but not chymase (41). In humans (18) and baboons (42) only a single *chm* gene can be identified, while rats and mice have multiple *chm* genes (43-48). Five *chm* genes have been identified in mice and two in rats. In rats, both *chm* genes are of the β-subtype, whereas four of the five mouse *chm* genes are of the β-subtype and one is of the α-subtype. Recent studies by Reynolds et al (49) indicate distinct patterns of expression of various chymases in different mouse mast cell populations.

The coding (more than 95%) and the 5′-flanking regions (−480 to +1, more than 92%) of the human and baboon *chm* gene are highly homologous (30,42). The 5′-flanking region of these primate *chm* genes contains a TATA box and a CAAT box. These features are typical of eukaryotic promoters. Our in vitro transcription studies have indicated that a GATA binding motif located −441/−417 bp upstream of the transcription-initiation site is an important *cis*-acting positive regulatory element in the baboon *chm* gene (42). In contrast, Sarid et al (50) have shown that expression of the rat *chm2* gene, encoding a β-chymase, is regulated by an enhancer element that contains regions of homology to pancreatic protease core enhancer elements. The rat *chm2* gene 5′-flanking region is highly homologous to equivalent regions of other β-chymases encoding *chm* genes (46), eg, the mouse *chm4* and *chmL* genes, but differs markedly from the 5′-flanking regions of the α-chymase encoding *chm* genes of baboon and human. It is possible that differences in the mechanism of transcriptional activation of α- and β-subtypes of the *chm* gene may have evolved to complement the changes in substrate specificity between these chymase isoenzymes.

EVIDENCE FOR CHYMASE-DEPENDENT ANGIOTENSIN II FORMATION IN PRIMATES

The immunocytochemical studies on localization of chymase in the heart and vessels (18) suggest that chymase plays a significant role in the interstitial formation of angiotensin II. However, since mast cells are major sites of elaboration of chymase and procurement of tissues could easily lead to mast cell degranulation and release of chymase, the possibility exists that in vivo chymase-dependent angiotensin II formation may be much less significant than that inferred from immunocytochemical studies or studies using isolated tissues. In vivo pharmacological studies with angiotensin I in the presence of ACE inhibitors are one way to show the importance of an alternative pathway to ACE in angiotensin II formation (14). However, in these studies the possibility exists that, even in the presence of high concentrations of ACE inhibitor, tissue ACE is not fully inhibited or that angiotensin I has intrinsic activity that allows a direct response without conversion to angiotensin II. These studies also do not rule out the possibility that nonspecific carboxypeptidases, rather than chymase, can convert angiotensin I to angiotensin II (51).

Hoit et al (28) used a novel approach to show that chymase-dependent angiotensin II formation can occur in conscious baboons. This approach uses a selective angiotensin II-containing substrate, [Pro^{11}, $DAla^{12}$]angiotensin I, which is an inactive precursor that yields angiotensin II upon incubation with chymase but not ACE. [Pro^{11}, $DAla^{12}$]angiotensin I has a 100-fold lower intrinsic activity for the angiotensin II receptor than angiotensin I. In in vitro biochemical studies, chymase but not ACE converted with [Pro^{11}, $DAla^{12}$]angiotensin I to angiotensin II (22). Using isolated human cardiac trabeculae, we have shown that [Pro^{11}, $DAla^{12}$]angiotensin I produces a positive inotropic response that can be inhibited by angiotensin II receptor

blockade (22). Infusion of [Pro^{11}, DAla^{12}]angiotensin I in conscious baboons produces hemodynamic and left ventricular functional changes consistent with systemic arterial vasoconstriction (28). Identical hemodynamic responses to [Pro^{11}, DAla^{12}]angiotensin I infusion, before or after pretreatment with captopril, indicate the selectivity of the response. Because chymase-like activity is present in several baboon tissues, including the heart and the aorta, these data suggest that the hemodynamic effects of [Pro^{11}, DAla^{12}]angiotensin I are the result of functional chymase-like activity in baboon tissues. The use of a chymase-selective substrate and of a conscious baboon model for demonstrating chymase-dependent angiotensin II formation circumvents potential problems related to the premature release of chymase from mast cells due to anesthesia or tissue handling. However, definitive proof of chymase-dependent angiotensin II formation in vivo requires a demonstration of decreased angiotensin II production following the administration of a specific chymase inhibitor.

IMPLICATIONS OF THE CHYMASE-ANGIOTENSIN SYSTEM IN THERAPEUTICS

The relationship among the antihypertensive effects of ACE inhibitors, ACE inhibition, and plasma angiotensin II levels is complex (9,10). During chronic therapy with ACE inhibition, plasma angiotensin II levels return to normal, despite a continued antihypertensive effect. The dissociation between plasma angiotensin II activity and the antihypertensive effect of ACE inhibitors may be related in part to the ability of ACE inhibitors to influence plasma levels of a number of peptide hormones including bradykinin and substance P (52). Thus, chronic suppression of angiotensin II production by inhibiting tissue chymase-like activity could be a useful adjunct to ACE inhibitors in the treatment of systemic hypertension.

ACE inhibitors are also used widely in the treatment of heart failure. The chymase-angiotensin system could allow continued angiotensin II formation in tissues despite effective ACE inhibition. This may be particularly important in congestive heart failure. In the presence of ACE inhibitors, resulting high levels of angiotensin I may allow a substantial level of angiotensin II formation in chymase-containing tissues such as the heart. Because cardiac angiotensin II participates directly in ventricular hypertrophy through its growth-promoting effects (53,54), inhibition of both chymase and ACE activities may be more beneficial than ACE inhibition alone in the treatment of congestive heart failure.

Recently, the findings of Powell et al (55) implicated an involvement of angiotensin II in the vascular proliferative response by showing that ACE inhibition is highly effective in decreasing neointimal formation resulting from arterial balloon injury in rats. However, clinical trials have failed to demonstrate a beneficial effect of ACE inhibitors on coronary arterial restenosis after percutaneous transluminal coronary angioplasty (56). Although concerns regarding the adequacy of ACE inhibition in restenosis and regression trials in humans are appropriate, continued vascular angiotensin II production by the chymase-angiotensin system could explain the negative outcome of these studies. In this regard, it is interesting that Shiota et al (57) recently showed a marked increase in chymase mRNA (threefold) and chymase-like activity (22-fold) in balloon injured dog arteries. In another recent, preliminary study these investigators also showed that both an ACE inhibitor and an angiotensin II receptor antagonist can reverse the proliferative response to arterial injury in the rat. In the dog, however, only the angiotensin II receptor antagonist, and not the ACE inhibitor, is effective in inhibiting the proliferative response to arterial injury (58). Because the rat does not contain an angiotensin II-forming α-chymase as in the human, baboon and dog, it is tempting to speculate about a role for the vascular chymase-angiotensin system in the pathogenesis of coronary arterial restenosis after percutaneous transluminal coronary angioplasty.

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